Title: Contribution of the CesR-regulated genes *llmg0169* and *llmg2164-2163* to *Lactococcus lactis* fitness.

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Running title: Contribution of ***llmg0169*** **and** ***llmg2164-2163*** **to** ***Lactococcus lactis*** **fitness**

Abstract

Lactotoccus lactis is one of the main components of the starter cultures used in cheese manufacture. As starter, *L. lactis* must tolerate harsh conditions encountered either during their production in bulk quantities or during dairy products processing. To face these hostile conditions, bacteria monitor the environment and respond by modifying gene expression appropriately. Previous transcriptomic studies showed that the two component system CesSR is the main pathway that triggers the cell envelope stress response in *L. Lactis* treated with lactococcin 972 (Lcn972), a cell wall synthesis inhibiting bacteriocin. Among the CesR regulated genes, *llmg0169* and the operon *llmg2164-2163*, encoding proteins of unknown function, are among the highest up-regulated genes after activation of CesSR. In this study, we have assessed the contribution of these genes to the survival of *L. Lactis* to different technologically-relevant stresses. Overexpressing and knock-out mutants of the genes were generated and their viability to low pH, heat, freeze-drying, presence of NaCl, cell wall antimicrobials and lytic phages attack was compared to the wildtype strain. The genes *llmg0169* and *llmg2164-2163* contributed differently to *L. lactis* fitness. *L. lactis* Δ*llmg0169* was very sensitive to heat treatment while *L. lactis* Δ*llmg2164* was more sensitive to NaCl. Absence of both genes also compromised viability at low pH. On the contrary, higher expression levels of *llmg0169* and *llmg2164-2163*, up to 26- and 14-fold increase determined by qRT-PCR, respectively, did not enhance *L. lactis* survival in any of the above stressful conditions (heat, pH and NaCl) or after freeze-drying. All the mutants displayed a similar phage susceptibility profile. Overexpression of *llmg2164-2163* seemed to specifically protect *L. lactis* against the bacteriocin Lcn972 but not against other cell wall active antimicrobials. Based on our phenotypic analysis, the investigated genes are required to mount a proper response to guarantee survival of *L. lactis* under technologically-relevant stresses and their functionality could be a useful marker to select robust dairy starters.

Keywords

Dairy starters, *Lactococcus lactis*, stress response, strain fitness, CesSR

1. Introduction

Lactococcus lactis is widely used as the main component of the starter cultures in cheese manufacturing processes. Due to their role in food fermentation, *L. lactis* has been extensively studied and has become a model bacterium among lactic acid bacteria (LAB). The main function of the *L. lactis* as a starter is to produce enough lactic acid through the fermentation of lactose, the most abundant sugar in milk. This leads to a pH decrease important for milk clotting and for preventing spoilage and/or pathogen development. *Lactococci* also contribute greatly to the textural and organoleptic quality of the fermented products by the synthesis of flavour compounds and texturing agents. The hygienic quality of the fermented product is also enhanced by the production of several antimicrobial compounds, including bacteriocins (Mäyra-Mäkinen and Bigret, 2005). The fermentation process relies, basically, on the optimal performance of the starter. Accordingly, starter strains have been traditionally selected on the basis of their acidifying activity, proteolytic activity and phage and bacteriocin resistance. However, the strains selected for industrial purposes should also tolerate adverse conditions encountered either during starter handling and storage or during dairy products processing in which stresses such as heat, cold, acidity, and high concentration of NaCl are common (van de Guchte et al., 2002).

In order to survive in a complex and changing environment, prokaryotes must be able to adapt to the new, usually harsh, conditions. They often achieve this by closely monitoring the environment and regulating gene expression accordingly. The stress response in LAB has been mainly approached by studying the effects on growth, the changing proteome and the genetic analysis of the genes involved (reviewed by Miyoshi et al., 2003; Sanders et al., 1999; van de Guchte, 2002). Common mechanisms with other Gram positives have been found such as the conserved heat shock proteins (the chaperones DnaK, DnaJ, GrpE, GroES and GroEL and the proteases Clp, HtrA, FtsH) and the cold shock proteins. Unique features have also

been observed as the low number of (alternative) sigma factors and two component systems (TCS) in *L. lactis* that indicates specific regulatory mechanisms (Bolotin et al., 2001).

As the most outer macrostructure in the cell, the cell wall is the major sensory interface between the cell and the environment. It is needed to maintain cell shape, counteract the inner osmotic pressure and it is the anchoring structure for many enzymes (Delcour et al., 1999; Desvaux et al., 2006). The cell wall is, therefore, crucial for survival and monitoring its integrity is of paramount importance. Previous transcriptomic analyses of *L. lactis* treated with lactococcin 972 (Lcn972), a bacteriocin that inhibits cell wall synthesis, revealed the TCS named CesSR as the main pathway to activate cell envelope stress response (Martínez et al., 2007). CesSR is homologous to the TCSs LiaSR (Mascher et al., 2004) and VraSR of *Bacillus subtilis* and *Staphylococcus aureus* (Kuroda et al., 2003), respectively. Both TCSs sense cell envelope stress.

The CesR regulon is composed of 21 genes identified by the presence of the CesR box in their promoters (Martínez et al., 2007). After activation of CesSR by Lcn972 treatment, the gene *llmg0169* and the operon *llmg2164-2163* were among the highest up-regulated genes, up to 300 and 21 times, respectively. *llmg0169* encodes a putative membrane protein of 79 amino acids with 3 transmembrane domains and homologus proteins are only found within *L. lactis*. *llmg2163* also encodes a putative membrane protein of 154 amino acids that holds at its N-terminus a PspC domain (Pfam04024) found in putative stress-responsive transcriptional regulators. Psp proteins are involved in response to extracytoplasmic stress and protect the cells probably by maintaining the integrity of the cytoplasmic membrane (Darwin, 2005). The other gene, *llmg2164*, specifies for a putative 370-amino acids conserved cytosolic protein (COG3595) of unknown function.

In this work we have generated loss-of-function and overexpressing mutants of *llmg0169* and *llmg2164-2163* to further characterize their contribution to the survival of *L. lactis* to the

stress often encountered during dairy processing. Namely, the survival at low pH, to heat treatment, freeze-drying, growth in the presence of NaCl, and resistance to cell wall antimicrobials and lytic phages has been determined.

2. Materials and Methods

2.1. Bacterial strains, culture conditions and growth parameters.

Strains and plasmids used in the present study are listed in Table 1. *L. lactis* strains were grown in M17 (Scharlau, Barcelona, Spain) plus 0.5 % glucose (GM17). When appropriate, the antibiotics (Sigma, Madrid, Spain) chloramphenicol (10 µg/ml) and erythromycin (5 µg/ml) were added. *E. coli* strains were used as intermediate cloning host and grown in 2xYT (Sambrock et al., 1989) at 37 ºC with shaking. When needed, ampicillin (Sigma) was added at 100 µg/ml. Solid medium for plating was supplemented with 2% agar. All experiments were started with an overnight culture (16-18 h, 30 ºC) prepared by resuspending a colony in the specific media. Growth was monitored by following the optical density at 600 nm (OD_{600nm}) in a Benchmark Plus Microplate Spectrophotometer (BioRad Laboratories, Hercules, Ca., USA) at 30 ºC, every 15 min for 8-9 h. Overnight cultures were diluted at an OD_{600nm} of 0.02 measured in a Biophotometer (Eppendorf, Spain) and 200 µl were placed on the microtitre plate. Specific maximal growth rates (μ_{max}) were calculated through linear regressions of the plots of $\log(OD_{600nm})$ versus time during the exponential growth phase and the generation time (g) determined from the relation specific growth rate $\mu = \ln(2)/g$.

2.2. Construction of llmg0169 and llmg2164-2163 L. lactis mutants.

Overexpressing mutants were obtained by cloning a DNA-fragment housing the genes and their promoter regions. These fragments were generated by PCR using the primers listed in Table 2. Total DNA of *L. lactis* NZ9000 was used as DNA template. A 940 bp amplicon containing *llmg0169* was obtained by using PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Buckinghamshire, UK) in a final volume of 25 µl. The program was: 95 ºC for 5 min; 30 cycles of 95 ºC for 45 s, 60 ºC for 1 min and 72 ºC for 45 s; and 72 ºC for 10 min. The PCR product was first cloned in pCR 2.1 (Invitrogen, Barcelona, Spain), cut out as a 1

kbp *Eco*RI fragment and cloned in the low-copy-number plasmid pIL252 to give pBL36 (Table 1). The 2.4 kbp amplicon containing *llmg2164-2163* was obtained using Expand Long Template PCR system (Roche, Mannheim, Germany). The PCR reaction was carried out in 1x PCR buffer containing dNTPs 1.4 mM, 0.3 µM of each primer and 3.75 U of Expand in a 50 µl reaction volume. The PCR program was: 94 ºC for 9 min; 30 cycles of 94 ºC for 30 s, 60 ºC for 30 s and 68 ºC for 3 min; and 68 ºC for 10 min. The PCR product was cloned in pCR 2.1, digested with *Sac*I and the 2.4 kbp *Sac*I fragment was cloned in pNZ124 (Table 1) to obtain pBL37. PCR reactions were performed in a MyCycler Thermal Cycler (BioRad Laboratories). Amplified products were visualized by agarose gel electrophoresis, and fragments were purified by using the Perfectprep Gel cleanup kit (Eppendorf) in accordance with the manufacturer recommendations. All the cloned fragments were confirmed by DNA sequencing. The non-polar and in-frame deletion of *llmg0169* was generated by cloning the upstream and downstream flanking regions, previously amplified by PCR using the primer pairs P1_*llmg0169*/P2_*llmg0169* and P3_*llmg0169*/P4_*llmg0169*, respectively (Table 2). Both fragments were cloned sequentially into pCS1966 (Table 1) using *Xba*I/*BamH*I for the upstream and *BamH*I/*Xho*I for the downstream fragments, respectively. All cloning steps were performed in *E. coli* DH5α. Transformation, integration of the resulting plasmid into *L. lactis* NZ9000, and its subsequent excision from the chromosome was performed and monitored as described (Solem et al., 2008). For *llmg2164* gene inactivation, a strategy based on gene disruption by single crossing-over (SCO) plasmid insertion was used. An internal fragment of *llmg2164* was PCR amplified from genomic DNA of *L. lactis* MG1363, the parent of *L. lactis* NZ9000 (Kuipers et al., 1998), using the primers ythC1 and ythC2 (Table 2). The PCR product was digested by *Eco*RI and *Xma*I, ligated to the pRV300 plasmid (Table 1), previously digested with the same enzymes, and transformed into *E. coli* HB101. The obtained plasmid was transformed in *L. lactis* MG1363, and insertional SCO mutants of

llmg2164 were selected on plates containing erythromycin (5 µg/ml) and PCR verified using the following primers ythC4 (5'-aatggatttgatgcgtcgtg-3') and ythC3 (5'-cagctgtaccattaatgag-3').

2.3. Quantitative reverse transcriptase PCR (qRT-PCR).

Three independent overnight cultures of each overexpressing *L. lactis* mutants and their controls harboring the empty vectors were inoculated at 1% in GM17 broth containing the required antibiotic and grown at 30 ºC until an OD_{600nm} of 0.5. Total RNA was isolated using the illustra RNAspin Mini RNA Isolation Kit (GE Healthcare). The RNA concentration was determined by absorbance at 260 nm and 0.2 µg were used to generate cDNA with the iScript cDNA Synthesis Kit (BioRad). PCR amplification was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems, Warrington, UK). Primers used for qRT-PCR are listed in Table 2. Amplification was carried out in 25 µl containing 0.01 µg cDNA, 1x Power SYBR Green (Applied Biosystems) and each primer at a concentration of 0.28 µM. After incubation at 95 ºC for 10 min, amplification proceeded with 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. The efficiencies of the primer sets were measured using a dilution series of cDNA. The raw threshold cycle (Ct) values were converted to relative expression levels by the $2^{(-\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001) to quantify the relative gene expression. The expression levels of *llmg0169* and *llmg2164-2163* were normalized to the housekeeping gene *tuf* encoding the elongation factor Tu.

2.4. Tolerance to heat and low pH.

Aliquots of 200 µl of overnight cultures were centrifuged at 15,600 x*g* for 5 min. Cell pellets were washed with NaCl 0.5% (w/v), pelleted again and resuspended in 1.5 ml of the same solution (control). To determine survival after heat treatment, samples (1 ml) were

incubated at 50 ºC for 15 min. and placed on ice for 10 minutes prior to plating. Non-treated samples were used as controls. Serial ten-fold dilutions were done in Ringer saline solution. Appropriated dilutions were deep-plated in GM17 plus 1.5% agar for subsequent colony counting after 24 h incubation at 30 ºC. Survival percentage was calculated as CFU/ml of the treated sample divided by CFU/ml of the untreated. To determine survival at low pH, the pH of the NaCl 0.5% solution was adjusted to pH 2 with HCl 12 N before resuspending the cells. Control and treated cells were incubated at 30 ºC for 30 min. Serial dilutions were made in sodium phosphate buffer 0.05 M, pH 6.8, to neutralize the low pH. The survival was calculated as described above using LogCFU/ml units. The pH challenge was carried out with at least three independent cultures.

2.5. Tolerance to NaCl.

Overnight cultures were diluted in GM17 to an OD_{600nm} of 0.02. Aliquots of 100 µl were dispensed in microplates containing 100 µl of GM17 with NaCl at 0, 2, 4, 6, 8, 10 and 12% (w/v). Growth at 30 ºC was monitored following the OD_{600nm} in a Benchmark Plus Microplate Spectrophotometer (BioRad) every 15 min until the control cultures (without NaCl) reached an OD_{600nm} of 1 (approximately 5 h of incubation). Each strain was inoculated three times in each microplate and the assays were carried out twice. The relative growth was defined as the OD in the presence of NaCl divided by the OD of the control culture.

2.6. Freeze-drying.

Overnight cultures of the overexpressing mutants and the corresponding strains with the empty plasmids were inoculated at 1% onto pre-warmed GM17 with the corresponding antibiotic and incubated at 30 ºC for 6 h. Cells were cooled down on ice and harvested by centrifugation at 9,700 x*g* for 10 min at 4 ºC. The cells were washed with cold Ringer saline

205 solution and concentrated 10x in sterile skimmed milk reconstituted at 11%. Samples of 2 ml
206 were dispensed into glass vials and frozen at -80 ºC for 48 h. Samples were lyophilized for 48
207 h and kept at 4 ºC. Cell viability was analyzed along the experiment and samples were taken
208 just before freezing (reference), after freezing, after lyophylization and after 15, 30 and 120
209 days of storage at 4 ºC. Vials were allowed to thaw at room temperature for 20 min, and 2 ml
210 of sterile distilled water were added prior to plating the appropriate decimal dilutions. The
211 assay was carried out with two independent cultures.
212
213 *2.7. MIC determinations.*
214 The MIC (Minimum Inhibitory Concentration) of the cell wall antibiotics penicillin G,
215 vancomycin, ampicillin, cefotaxime, oxacillin, and imipenem was determined using Oxoid
216 M.I.C.Evaluator® strips (Oxoid, Basingstoke, UK) according to the manufacturer
217 instructions. Plates were incubated at 30 ºC for 24 h. The MIC of bacitracin (Sigma) and the
218 bacteriocins nisin (kindly supplied by Applin and Barret Ltd., UK) and Lcn972 was
219 determined by the broth microdilution method in GM17 as previously described (Martínez et
220 al., 2007). Lcn972 was purified from culture supernatants as described (Martínez et al., 2007).
221 The concentration range of the inhibitors was: nisin, 0.002 – 2.5 μg/ml; bacitracin, 0.15 – 10
222 μg/ml and Lcn972, 1.25 – 640 AU (Arbitrary Units)/ml).
223
224 *2.8. Phage susceptibility.*
225 Phage suspensions were obtained from filtered supernatants of *L. lactis* MG1614 cultures
226 infected and lysed by a single plaque of the phage c2 and two milk isolated phages 1168 and
227 9203, belonging to the c2 and 936 families, respectively (Madera et al., 2004). Plaque assays
228 were performed spreading on GM17 plates 100 μl of decimal dilutions of the phage
229 suspension in SM buffer (20 mg/L Tris HCl, 10 mg/L $MgSO_4$, 10 mg/L $CaCl_2$, 100 mg/L

230 NaCl, pH 7.5) and 3 ml of molten GM17 0.7% agar inoculated with 100 µl of the appropriate
231 overnight *L. lactis* culture. Plates were incubated at 30 ºC for 18 h. Efficiency of plaquing
232 (EOP) was determined using the wild type strain *L. lactis* NZ9000 as the reference strain.
233 EOP was defined as the phage titre on the test strain divided by that on the reference strain.
234 Each phage suspension was plated in triplicate.
235
236 *2.9. Statistical analyses.*
237 The results were compared by one-way ANOVA analysis using Statistical Package for the
238 Social Sciences (SPSS) 15.0 software for Windows (Chicago, IL, USA).

3. Results

3.1. Stability and growth behaviour of llmg0169 and llmg2164-2163 L. lactis mutants.

These genes and their promoter regions were cloned in multicopy plasmids to obtain the overexpressing mutants (Table 1). After several attempts, it was not possible to clone *llmg0169* into the high copy number plasmid pNZ124 and the gene could only be maintained in pIL252. Despite of this, the expression level of *llmg0169*, determined by qRT-PCR, was over 26-fold higher compared to the parental *L. lactis* NZ9000, whereas the operon *llmg2164-2163* was 14-fold. All the mutants grew normally under the laboratory conditions in GM17 broth and no macroscopically relevant features were noticed in the isolated colonies growing on plates. The growth parameters under laboratory conditions are shown in Table 3. The mutant 0169+ grew slightly faster (p <0.05) than the parental strain, which might indicate a positive effect of the overexpressed gene. No relevant differences were observed among any of the other mutants.

Another parameter to evaluate the impact of the mutations in *L. lactis* was to assess the stability of the plasmids, in the case of the overexpressing mutants, and the reversion rate of the insertional knock-out Δ2164. The strains were subcultured in the absence of the antibiotic for several generations and the percentage of the remaining antibiotic resistant cells was calculated by replicating colonies in the presence or absence of antibiotic (Table 3). Both *llmg2164-2163* mutants were readily stable up to 100 generations but, further on, the plasmid pBL37 was lost notably faster than the parental empty plasmid pNZ124. On the contrary, *L. lactis* Δ2164 was very stable and no revertants to the wild type genotype were detected for up to 200 generations. The mutant *L. lactis* 0169+ was the most unstable. However, the presence of *llmg0169* appeared to improve the stability of pIL252, highlighting once more a positive role of the gene in the growth of *L. lactis* under laboratory conditions.

3.2. Tolerance to technologically relevant stresses.

As a dairy starter, *L. lactis* is often exposed to low pH, high salt concentrations and, in particular circumstances, to mild heat treatment. The survival of the *L. lactis* mutants in acidic conditions and after a heat shock of 15 min at 50 ºC is displayed in Fig. 1. After the exposure to pH 2, the survival of both knock-out mutants was significantly lower ($p<0.05$) than the wild type strain. However, overexpression of *llmg0169* and *llmg2164-2163* did not increase the tolerance to acidic conditions (Fig. 1A). The strains carrying the empty plasmids pNZ124 and pIL252 were also processed in the same way and no differences to NZ9000 were observed (data not shown). The survival after heat treatment was also clearly compromised in the knock-out mutants (Fig. 1B). Remarkably, the mutant Δ0169 was extremely sensitive and showed only 10% survival after the treatment versus 75% of the wild type strain. No significant differences ($p>0.05$) were observed between the wild type and the overexpressing mutants.

The ability of the mutants to grow in the presence of increasing concentrations of NaCl was determined and compared to the wild type strain (Fig. 2). None of the strains were able to grow at NaCl concentration over 3%. Slight differences were seen at lower NaCl concentrations. At NaCl 2% and 1%, both *llmg0169* mutants grew slower than the wild type strain ($p<0.05$). Similarly, *L. lactis* Δ2164 was also more sensitive ($p<0.001$) to the presence of NaCl. The overexpresing mutant 2164-2163+ behaved like the wild type strain.

3.3. Survival after freeze-drying.

The most common preserving and delivery strategy of dairy starters is either frozen or lyophilised. The viability after both processes and during the storage of the lyophilised powder at 4 ºC was determined in the overexpressing mutants. As controls, *L. lactis* NZ9000 transformed with the corresponding empty plasmids were used. The results are shown in Fig.

289 3. After freezing the cells at -80 ºC and prior to lyophylization, there was a slight decrease of
290 the viability of the cultures, except for the strain *L. lactis* NZ9000/pIL252 which was
291 seriously affected. We presume that this sharp loss of viability was due to the instability of the
292 plasmid pIL252, rather than to a higher intrinsic susceptibility of the strain to low
293 temperatures, because the viable counts at this time point were done in GM17 plus antibiotic.
294 Therefore, only viable cells with the plasmid would have been counted. Nevertheless, it is
295 remarkable that the presence of *llmg0169* seemed to counteract plasmid loss even after
296 stressing conditions because the viability, in this case, was hardly affected. All the strains
297 were seriously damaged after lyophylization and only 30-50% of cells remained viable.
298 Viability decreased slowly during storage of lyophilised samples at 4 ºC to a 10% average of
299 viable cells after 120 days of storage. There was neither negative nor positive effect of any of
300 the mutations on the survival along the sampling period.
301
302 *3.4. Susceptibility to cell wall antimicrobials.*
303 The MICs of several cell wall active antimicrobials for the different *L. lactis* mutants are
304 summarized in Table 4. No differences over a factor of 2 were observed with any of the
305 antibiotics tested except for the mutants of *llmg2164-2163*. The knock-out mutant Δ2164 was
306 three to four times more resistant to penicillin G, oxacillin, and nisin, respectively, than the
307 wild type strain. The overexpressing mutant was more resistant to Lcn972, confirming a
308 direct role of these genes in the response to Lcn972 treatment. On the contrary, the
309 overexpression of *llmg0169*, also induced by Lcn972, did not result in an Lcn972 resistant
310 phenotype. To test if the resistance to Lcn972 displayed by the strain *L. lactis* 2164-2163+
311 was due to changes of the cell envelope, the adsorption of Lcn972 to the cells of the
312 *llmg2164-2163* mutants was compared. Irrespectively of their genotype, similar amounts of

313 Lcn972 (*ca*. 30%) were adsorbed by *L. lactis* cells in sodium phosphate buffer 50 mM, pH 6.8
314 (data not shown).
315
316 *3.5. Phage susceptibility*
317 Phages constitute one of the major leading causes of food fermentation failures. Since the
318 analysed genes specify membrane proteins, which may influence the initial steps of phage
319 infection as host recognition, the phage susceptibility pattern of the *L. lactis* mutants was
320 assessed (Table 5). The EOPs were slightly lower for the three different phages on all the *L.*
321 *lactis* mutants but no correlation to the overexpression or absence of the genes was observed.
322 Thus, the putative changes on the cell envelope due to the corresponding proteins do not seem
323 to interfere widely with phage infection.

4. **Discussion**

Consistent food fermentations rely largely on the optimal performance of the starters. Tolerance to technological processes such as starter production, conservation, and viability in the fermented food product are some of the selection criteria for robust and trustworthy starters. Special efforts have been made to understand the molecular mechanisms underlying stress response in lactic acid bacteria (reviewed by Sanders et al., 1999; Duwat et al., 2000; van de Gutche et al., 2002). Studies on the stress response and the effect of mutations in the global stress regulators have lately been addressed by global transcriptomic and proteomic analyses (Budin-Verneuil et al. 2007; Martínez *et al*., 2007; Sánchez et al., 2005; Zomer et al*.*, 2007; Zotta et al., 2008). However, many of the regulated genes which respond to a particular stress encode proteins of unknown function whose role in the stress response remains elusive. This is the case of the genes *llmg0169* and *llmg2164-2163* selected in this work, which belong to the cell envelope stress regulon and thereby, they are regulated by CesR. Besides induction after bacteriocin treatment (Martínez et al., 2007), the CesR regulator was shown to be activated in the early response to acid stress (Zomer et al., 2007) and by lysozyme treatment (Veiga et al., 2007). Other CesR orthologs were shown to respond to cell wall active antimicrobials and to protein secretion stress (Jordan et al., 2008).

Based on the relevance of the CesR regulator in *L. lactis* stress response, overexpressing and loss of function mutants of the highest upregulated genes, *llmg0169* and *llmg2164-2163*, were analysed. The mutations did not noticeably affect the growth parameters indicating that they are dispensable and their overexpression does not critically hinder *L. lactis* fitness under laboratory conditions. However, the lower mRNA levels of *llmg2164-2163* compared to *llmg0169* mRNA, despite of being cloned in a higher copy plasmid and containing a similar promoter region, denote that higher expression levels of *llmg2164-2163* and, likely, a higher production of the corresponding proteins, are not well tolerated by *L. lactis*. This hypothesis

349 was also supported by the faster loss of the plasmid pBL37 compared to the empty plasmid.
350 On the contrary, *llmg0169* seems to stabilise pIL252 shown to be segregationally unstable
351 (Simon and Chopin, 1988). However, higher expression levels are also apparently toxic to *L.*
352 *lactis* since this gene could not be cloned in pNZ124.

353 Our study shows that the investigated genes contribute differently to survival of *L. lactis*
354 facing several technological stresses in spite of being under the control of the same regulator.
355 The strain *L. lactis* Δ0169 was very sensitive to heat treatment whereas *L. lactis* Δ2164 was
356 more affected than the others by the presence of NaCl. Previous work has shown that mutants
357 lacking the common response regulator CesR were particularly susceptible to osmotic stress
358 but not to heat shock (O´Connell-Motherway et al., 2000). This divergence could be based on
359 the different experimental conditions to evaluate survival but it could reflect also that CesR is
360 not the only regulator of *llmg0169* and the gene could be regulated by alternative pathways.
361 On the other hand, both knock-out mutants were more susceptible to acid stress than the
362 parental strain. Although the viability was not dramatically compromised, both genes seem to
363 contribute to the survival to low pH and confirm previous *L. lactis* transcriptomic analysis in
364 which *llmg0169* and *llmg2164-2163* were detected as up-regulated genes in the late response
365 to lactic acid exposure (Zomer et al., 2007).

366 The viability of the overexpressing mutants after freeze-drying was similar to the parental
367 strain. According to the literature (Santivarangkna et al. 2008), the very low survival after
368 freeze-drying was clearly due to the drying step since the viability was maintained after
369 freezing at -80 ºC. The only exception was the control strain carrying the plasmid pIL252
370 whose viability was seriously compromised after freezing. As described in the result section,
371 we presume that loss of viability was likely due to the high instability of pIL252. Noteworthy,
372 the presence of *llmg0169* increased the stability of the plasmid under freezing conditions.
373 Regardless the strain, the total loss of viability of *L. lactis* after freeze-drying was rather high.

374 This could be related to the absence of any cryoprotector (besides milk) in our preparations.
375 Cryoprotectors are often added to the cell suspension prior to freezing and enhance cell
376 viability (Cárcoba and Rodríguez, 2000).

377 In spite of the negative impact of the loss-of-function mutations, overexpression of the
378 genes did not have any clear positive effect on survival of *L. lactis* under the stress conditions
379 discussed above. This was not the case of other CesR regulated gene which has been
380 previously characterized. For example, cloning of a DNA fragment containing the CesR-
381 regulated *spxB* gene in a multicopy plasmid conferred resistance to lysozyme in *L. lactis*
382 (Veiga et al., 2007). It has also been described that lactic acid bacteria overproducing the
383 chaperon protein GroESL moderately increased tolerance to heat, drying and high osmotic
384 pressure (Corcoran et al., 2006; Desmond et al., 2004).

385 The only phenotype clearly linked to the overexpressing mutants was the resistance to the
386 bacteriocin Lcn972 of the strain *L. lactis* 2164-2163+. Protection against Lcn972 seems not to
387 be related to changes in the cell surface that reduce adsorption of the protein. Absence of
388 drastic changes at the surface has also been confirmed by the similar phage susceptibility
389 pattern shown by the *L. lactis* mutants. Lcn972 inhibits cell wall biosynthesis at the division
390 septum by binding to lipid II (Martínez et al., 2008). It is possible that the products of these
391 genes protect the cell from the lethal effect of a weaker cell wall. However, no resistance to
392 other cell wall active antibiotics has been observed and the protection seems to be Lcn972
393 specific.

394 According to our phenotypic analysis, the products of *llmg0169* and *llmg2164-2163* are
395 necessary to achieve a proper response to several technological stresses in *L. lactis* and their
396 functionality could be used as a selection marker for strong and robust starters. It has also
397 been shown that a functional *llmg0169* is crucial to ensure viability of *L. lactis* after a mild
398 heat shock. However, as far as we know, this gene has never been identified among heat

shock activated genes in *L. lactis.* A deeper insight into the regulation and localization of this putative protein could shed new lights into its mode of action and the complex stress response in *L. lactis.*

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Figure legends

Figure 1. Survival of *L. lactis* mutants after exposure to acidic conditions (A) and after incubation at 50 ºC for 15 min (B). Survival was calculated as Log(CFU/ml) of treated cultures divided by Log(CFU/ml) of untreated cultures and expressed as % in (A), due to the low counts after exposure to low pH, and as CFU/ml of treated cultures divided by CFU/ml of untreated cultures and expressed as % in (B). Values are the mean of three independent experiments. Error bars indicate standard deviations. WT: wild type strain *L. lactis* NZ9000.

Figure 2. Growth of *L. lactis* mutants in the presence of NaCl. Relative growth was referred to a control culture in the absence of NaCl and measured as OD_{600nm}. WT: wild type strain *L. lactis* NZ9000.

Figure 3. Survival of *L. lactis* mutants after lyophylization and storage at 4 ºC. Cultures were grown until the end of the exponential phase and concentrated in skimmed milk. Viable counts (CFU/ml) at this stage were taken as 100 %. AF, after freezing at -80 ºC for 48 h; AL, after lyophylization; pIL252, *L. lactis* NZ9000/pIL252 (white bars); pNZ124, *L. lactis* NZ9000/pNZ124 (grey bars); 0169+, *L. lactis* overexpressing *llmg0169* (vertical striped bars); 2164-2163+, *L. lactis* overexpressing *llmg2164-2163* (horizontal striped bars).

Table 1.

Bacterial strains and plasmids.

Strain/plasmid	Description [a]	Reference
Strains		
Lactococcus lactis		
NZ9000	plasmid-free, carrying *pepN*::*nisRK*	Kuipers et al., 1998
0169+	NZ9000 overexpressing *llmg0169*	This study
Δ0169	NZ9000, *llmg0169* deleted	This study
2164-2163+	NZ9000 overexpressing *llmg2164-2163*	This study
Δ2164	MG1363, *llmg2164* disrupted by pRV300	This study
Plasmids		
pCR2.1	Cloning of PCR products, Ap^r	Invitrogen
pRV300	pBluescript derivative, Em^r	Leloup et al., 1997.
pCS1966	Integrative plasmid, Em^r	Solem et al., 2008
pIL252	Low copy plasmid, Em^r	Simon and Chopin, 1988
pNZ124	High copy plasmid, Cm^r	Plateeuw et al., 1994
pBL36	pIL252, *llmg0169* in *Eco*RI (940 bp)	This study
pBL37	pNZ124, *llmg2164-2163* in *Sac*I (2400 bp)	This study

[a] Ap, ampicillin; Em, erythromycin; Cm, chloramphenicol

Table 2.

Primers used in this study.

Primer	Sequence [a] 5'-3'	Description
5' *llmg0169* F	TGATAATGTCGCTCCTAATGC	Cloning *llmg0169*
3' *llmg0169* R	CTgaGCTCATGACGCGATG (*Sac*I)	
5' *llmg2164* F	AGGAATCGCTACAGATTTGAC	Cloning *llmg2164-2163*
3' *llmg2164* R	AGTGAgCtCCATATTTCTGCTGG (*Sac*I)	
P1_*llmg0169*	AAAATtctagAGACAGCTGGCTTGCTAATCATCC (*Xba*I)	Cloning of *llmg0169* adjacent regions into pCS1966
P2_*llmg0169*	AAGCGggatccGTACGATTATTCCTGACAAAATTCC (*BamH*I)	
P3_*llmg0169*	AGTCCggatccTACTTGGTAAAATGAAAAATTAAGCAGC (*BamH*I)	
P4_*llmg0169*	GTGCTctcgagGAGTAGTAGGCCACCGAGTAACC (*Xho*I)	
ythC1	CAAAAGAATTCTCATATACAGAC	Disruption of *llmg2164*
ythC2	ATGATGcccgggCTGCAGAAATTCGACCATTT (*Xma*I)	
QRT F *0169*	TTTGTCAGGAATAATCGTACTTGGAG	qRT-PCR for *llmg0169*
QRT R *0169*	CCATCAAACGAATGACCAACAGAATG	
QRT F *2164*	GATAGAATAATGGATTTGATGCGTCG	qRT-PCR for *llmg2164-2163*
QRT R *2164*	TTCATCCGAAGTTTCTGAATCAACTG	
Tuf F	GGTAGTTGTCGAAGAATGGAGTGTGA	qRT-PCR internal control
Tuf R	TAAACCAGGTTCAATCACTCCACACA	

[a] Changes introduced to generate restriction sites (underlined and shown in brackets) are indicated in lowercase letters.

Table 3.

Growth parameters and stability of *llmg0169* and *llmg2164-2163 L. lactis* mutants

L. lactis	Growth parameters [a]		Stability (%) [b]	
	μ_{max} (h^{-1})	g (h)	100 gen.	200 gen.
NZ9000	1.02±0.02	0.99±0.03	NA	NA
0169+	1.09±0.01	0.91±0.01	53.6 (18.5)	0.60 (0.0)
Δ0169	1.06±0.05	0.95±0.05	NA	NA
2164-2163+	1.01±0.05	1.00±0.05	92.2 (92.3)	1.8 (95.2)
Δ2164	1.06±0.03	0.94±0.03	100 (NA)	100 (NA)

[a] Growth parameters were estimated from 3 independent cultures.
[b] Stability was assessed after 100 and 200 generations (gen.) in the absence of the corresponding antibiotic and defined as the % of antibiotic resistant cells. The stability of the empty plasmid is shown in brackets. NA: not applicable.

Table 4.

MICs of several cell wall antimicrobials for *L. lactis* mutants.

L. lactis	Antibiotic MIC (μg/ml) [a]								
	CTX	AMP	VA	OX	P	IPM	BAC	Nisin	Lcn972 [b]
NZ9000	0.06	0.12	0.12	0.5	0.12	0.03	1.25	0.15	40
0169+	0.12	0.12	0.185	0.5	0.185	0.03	0.6	0.15	40
Δ0169	0.09	0.12	0.185	0.5	0.25	0.03	1.25	0.15	20
2164-2163+	0.09	0.12	0.185	0.5	0.185	0.03	0.6	0.07	160
Δ2164	0.12	0.12	0.185	1.5	0.375	0.03	0.6	0.6	20

[a] The MICs were determined with M.I.C.Evaluator® strips except for bacitracin (BAC), nisin and Lcn972 which were determined by the broth microdilution method. CTX, cefotaxime; AMP, ampicillin; VA, vancomycin; OX, oxacillin; P, penicillin G; IPM, imipenem.
[b] MIC in Arbitrary Units/ml.

Table 5.

Efficiency of plaquing (EOP) of lactococcal phages on *L. lactis* mutants.

L. lactis	EOP of bacteriophages [a]		
	c2	1168	9203
NZ9000	1.01±0.08	1.00±0.09	1.00±0.03
0169+	0.82±0.10	0.91±0.04	0.84±0.06
Δ0169	0.70±0.17	0.80±0.19	0.74±0.15
2164-2163+	0.83±0.11	0.67±0.02	0.61±0.06
Δ2164	0.90±0.19	0.68±0.04	0.61±0.02

[a] Bacteriophages 1168 and 9203 are natural isolates from milk belonging to the c2 and 936 families, respectively (Madera et al., 2004).

Fig. 1. Roces et al.

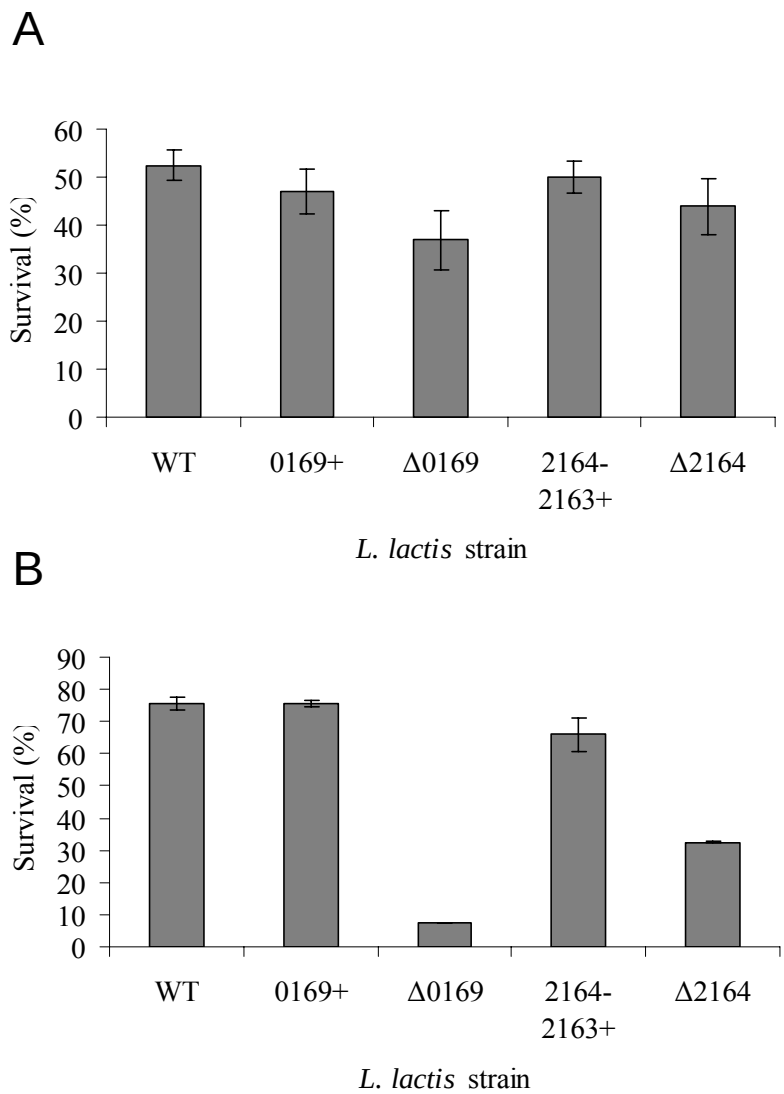

Fig. 2. Roces et al.

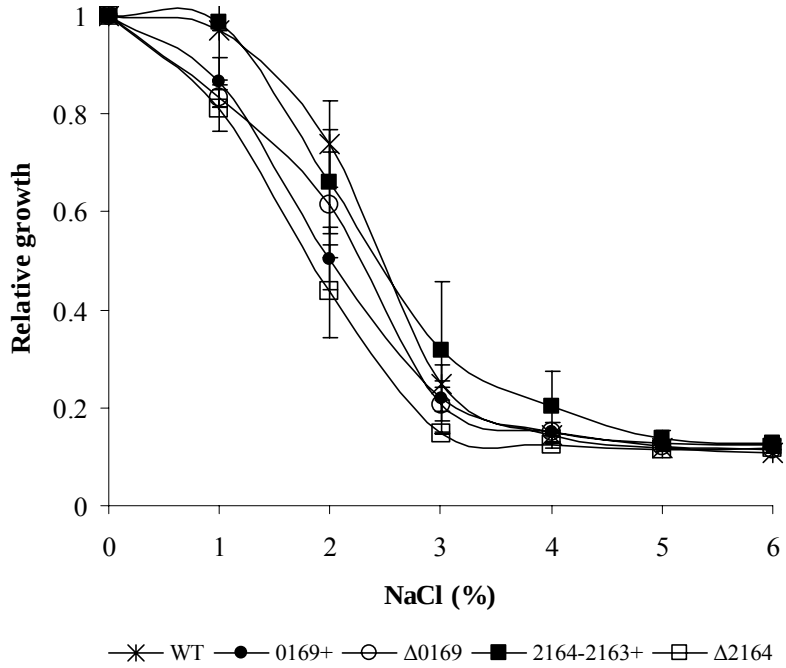

Fig. 3. Roces et al.